Cys-Loop Receptors

3256-Pos Board B411
Loop-C and a Cyclic Activation Scheme for Acetylcholine Receptor-Channels (AChRs)
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The 'capping' of loop-C at each transmitter binding site has been proposed to initiate the AChR channel-opening isomerization. We recorded single-channel currents from various loop-C constructs (adult mouse; both α-subunits, 188-198: VFYSCCPTTPY), both with and without ACh, to probe its role in binding vs. gating. One construct (11gly) had glycines at all positions, and others had n=9, 7, 5 or 3 glycines. The loop A mutation αA96H was added as a background to increase constitutive activity. 1) None of these constructs altered the unliganded gating activity. 2) The open-probability of the 11gly-construct was not increased by 1mM ACh. 3) Reverting only two amino acids (bold) in the 11gly-construct to tyrosines generated AChRs that were activated by ACh, again without altering unliganded gating. These results suggest that loop-C is mainly involved in agonist binding.
A sequential kinetic scheme has been used to describe brief and long unliganded openings in AChRs with background pore mutations (βδM2,9'-S). We compared this scheme with others using AChRs activated by low [ACh], in a construct that had only one functional binding site (αW149F+ββδ9'S+εP121R). The interval durations were fitted across-concentrations using three different kinetic schemes, each having four states (C, O, AC and AO). The best fit was obtained by using the full cycle (MWC model). The estimated increase in the gating equilibrium constant with ACh (~740-fold) was similar to that obtained previously by saturating the binding site (~690-fold). These results show that a cyclic scheme describes brief/long unliganded openings at low [ACh] and can be used to estimate the association/dissociation rate constants to the high-affinity, open transmitter binding site.

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Intrinsic Gating Energy of Fetal-Type Neuromuscular Acetylcholine Receptors
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Fetal-type neuromuscular AChRs are composed of $(\alpha 1)_2\beta\delta\gamma$ subunits, whereas adult-type have the stoichiometry $(\alpha 1)_2\beta\delta\varepsilon$. γ-AChRs are indispensable for functional synapse formation, and mutations in this receptor subtype have been associated with congenital myasthenia, multiple pterygium syndrome and premature death. Cellular responses to transmitter molecules are influenced by a receptor's unliganded gating energy, G_0. In mouse ε-AChRs, G_0=+8.4 kcal/mol (−100 mV). We estimated this intrinsic gating energy in γ-AChRs (single-channels, HEK cells), from measurements of the unliganded gating equilibrium constant E_0 (G_0=-0.59lnE_0). E_0 was extrapolated from observed E_0 values in γ-AChRs having different combinations of background mutations that increased constitutive activity. First, 12 different background mutations were characterized separately (by measuring the diliganded gating equilibrium constant with choline) and were found to alter only G_0 and not the energy from the agonist. The effects of the mutations in γ-AChRs were approximately the same as in ε-AChRs. The extrapolation was based on two assumptions, that the change in the diliganded gating equilibrium constant (E_2) is caused by only an equivalent change in G_0, and that the G_0 changes are independent. A log-log plot of E_0^{obs} vs. E_2^{mut}/E_2^{wt} resulted in straight line with slope 1.08 ± 0.09, validating the two assumptions. From the extrapolation, $E_0^{wt,\gamma} = 4.8\times10^{-8}$, or $G_0^{wt,\gamma}$ =+9.9 kcal/mol. This value is ~+1.5 kcal/mol larger than ε-AChR value. The inherent 'slower' kinetics of γ- vs. ε-AChRs may, in part, be attributed to the elevated intrinsic energy barrier of the gating transition state. The results are discussed in the light of a MWC thermodynamic cycle, to derive other equilibrium and kinetic parameters for γ-AChRs. This works lays the foundation for understanding the fetal-type AChR transmitter binding site.

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α-Conotoxin Regiia Targeting Nicotinic Acetylcholine Receptors: Mutagenesis Studies Improving Selectivity and Potency
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Nicotinic acetylcholine receptors (nAChR) play important roles in various physiological functions including pain, anxiety, fatigue, memory and learning. The α3β4 nAChR subtype has been implicated in various conditions, including lung cancer and nicotine addiction. Selective nAChR antagonists are invaluable for evaluating the functional roles of nAChR subtypes in the nervous system. α-Conotoxins that act as nAChR antagonists have been identified from the venom of predatory marine cone snails. We previously reported the discovery of a new α4/7-conotoxin, RegIIA, isolated from *Conus regius*. RegIIA inhibits acetylcholine (ACh)-evoked currents mediated by α3β2, α3β4 and α7 nAChR subtypes. RegIIA is the most potent α3β4 nAChR antagonist known, to date, with an IC_{50} of 48 nM (Franco, A., et al. 2012. Biochem. Pharmacol. *83*:419-426). This study aims to understand and improve RegIIA's selectivity profile at the α3β4 nAChR subtype. It uses alanine scan mutants of non-cysteine residues within loop 2 of RegIIA, which were synthesised using solid-phase peptide synthesis. These mutants were functionally tested on nAChRs expressed in *Xenopus* oocytes using two-electrode voltage clamp recording. Of these mutants, [N11A] and [N12A] RegIIA exhibited a three-fold increase in selectivity for α3β4, compared with the α3β2 nAChR subtype. Molecular dynamics simulations of RegIIA bound to the ACh binding pocket of the α3β4 nAChR subtype revealed crucial molecular interactions, including residues of the ''aromatic cage'' at the α3(+) principal face (W174, Y215, Y222) as well as the β4(-) complementary face (W79, K81, R135). This study extends our understanding of RegIIA interactions with various nAChR subtypes and elucidated the key residues involved on both the peptide and the receptor binding site. Furthermore, we have obtained valuable information about the future design and development of α3β4-selective drugs that could target lung cancer and nicotine addiction.

3259-Pos Board B414
Energetic Changes in the β Subunit during Gating of the Muscle Nicotinic Receptor
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The transmitter-binding sites in the adult muscle-type nicotinic receptor are located at the interfaces between the α subunit and the δ or ε subunits, while the β subunit does not contribute to binding. The β subunit is termed a "structural" subunit as a result. We made mutations to 27 residues in the mouse muscle β subunit (2 to 4 mutations at each location) to measure the consequences on the channel opening and closing rates. We analyzed the results in terms of the effect on the ratio of the opening to closing rates (θ) and, when sufficiently large changes occurred, on the slope of the logarithmic relation between the opening rate and θ (φ). φ can be interpreted in terms of the timing during channel activation when a given residue makes an energetic contribution to gating. We examined 12 positions in the extracellular domain (ECD) of the β subunit, chosen because mutations to homologous residues in other subunits had large effects on θ. In contrast, mutations at the locations in β produced small changes in gating. We examined 15 positions in the second transmembrane (TM2) and TM2-TM3 linker regions. The majority of locations showed larger changes in θ(typically $>$ 1 kcal/mole). Estimates of φ were consistent with the idea that the energetic contributions from residues in the β subunit occurred later than for homologous residues in the α subunit but before residues in δ.
Overall, the data suggest that the ECD and the interface between the ECD and TM domains of β make little contribution to the energetics of gating. However, channel-lining residues do contribute, although the data suggest that the timing is later than for the α subunit. (NS22356 & NS72770)

3260-Pos Board B415
Effect of Membrane Hydrophobic Thickness on the Uncoupling of Binding/Gating in the Nicotinic Acetylcholine Receptor
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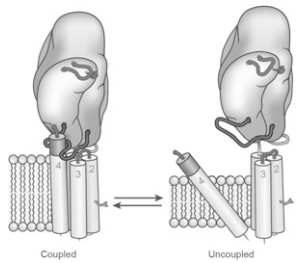
The effects of allosteric modulators on the nicotinic acetylcholine receptor (nAChR) are usually interpreted in terms of a model involving pre-existing resting, open, and desensitized conformations. The nAChR also adopts a lipid-dependent non-activatable conformation, referred to as the ''uncoupled'' state. Neither the global secondary structure nor the thermal denaturation of the nAChR is greatly affected by uncoupling, although uncoupling leads to an increase in peptide hydrogen exchange suggesting that previously buried peptide hydrogens become exposed to aqueous solvent. We propose that uncoupling results from a weakening of the physical interactions at the interface between the agonist binding and transmembrane pore domains because of lipid-dependent alterations of M4/Cys-loop interactions. As a preliminary test of this model, we reconstituted the nAChR into phosphatidylcholine

(PC) membranes with varying hydrophobic thicknesses. While the nAChR in each PC membrane adopts an uncoupled conformation, long acyl chain lipids promote very slow agonist-induced conformational transitions to the desensitized state. Hydrophobic thickness likely influences the ability of the nAChR to undergo agonist-induced conformational transitions by promoting α-helix/α-helix interactions, which appears to lower the activation energy barriers between uncoupled and coupled conformations.



3261-Pos Board B416

Alpha7 Nicotinic Receptors: Intrinsic Kinetics and Modulation by PNU 120596

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We investigated the kinetics of choline evoked currents, and the effect of the positive modulator PNU 120596 on alpha7 nicotinic acetylcholine receptors expressed by GH4C1 cells (obtained from Siena Biotech SpA) in whole-cell and outside-out patch-clamp experiments. using a theta-tube system for rapid application of agonists, we measured the dependence of current kinetics on the solution exchange rate. By extrapolation and by kinetic modeling we estimated the intrinsic kinetics of the receptor: what would be the amplitude and kinetics of choline-evoked currents at instantaneous agonist application. In the presence of PNU 120596 the single channel mean open time is drastically prolonged, this allowed us to determine the ratio of simultaneously open channels using nonstationary fluctuation analysis. By determining the approximate number of channels in a patch, we could determine the peak open probability of 10 mM choline-evoked currents in the absence of the modulator (0.0333 ± 0.0056), as well as the open probability in the presence of 10 mM choline and 10 μM PNU 120596 (0.632 ± 0.065). We performed kinetic experiments to determine the affinity of PNU 120596 to three different conformational states of the receptor: resting state, desensitized state and a slowly developing second desensitized state. We found that PNU 120596 was ineffective at both the resting and the slow desensitized states, while it bound to the desensitised state and re-activated the receptors. We investigated the nature of cooperativity between the agonist and the modulator. We found that while the agonist increases the apparent affinity of the modulator only by inducing desensitized conformation (which is preferred by the modulator), the modulator induces a true increase of agonist affinity probably by allosterically affecting the conformation of the agonist binding site itself.

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Anesthetic Binding Sites within the Transmembrane Domain of the α7 Nicotinic Acetylcholine Receptor

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General anesthetics inhibit the α7 neuronal nicotinic acetylcholine receptor (nAChR) with different sensitivities, but the location of anesthetic binding sites remains unclear. Here we used high-resolution solution NMR to study the interaction of general anesthetics ketamine and halothane with the transmembrane domain (TMD) of the human α7 nAChR. The TMD was expressed in *E. coli* and purified into detergent LDAO micelles. The specific residues involved in anesthetic binding were identified by anesthetics-induced chemical shift changes in ^{1}H-^{15}N HSQC NMR spectra. Ketamine binds to an intrasubunit site involving residues of all four helices at the intracellular end of the TMD. Two halothane interaction sites were detected. The first site was located near the intracellular end of the TMD, similar to the ketamine site, but involved only residues in TM1 and TM2. The second halothane site was formed by residues in the middle of TM1 and TM3. This site was not observed for ketamine binding, probably because ketamine was too large to penetrate deep into the TMD. Interestingly, unlike the previous finding in the α4β2 nAChR, anesthetic binding to the extracellular end of the TMD was not observed in the α7 nAChR. This difference may be responsible for their distinct sensitivity to halothane. In addition, halothane modulated motion less in α7 than in α4β2 on the micro- to milli-second (μs-ms) timescale, as demonstrated by changes in peak intensity, line width, and peak splitting for some residues. This work was supported by NIH grants: R01GM066358, R01GM056257, and R37GM049202.

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Probing the Trans-Membrane Domain of GLIC, a Prokaryotic Ligand-Gated Ion Channel

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The Gloeobacter ligand-gated ion channel, GLIC, has up to 28% sequence identity with eukaryotic Cys-loop receptors, and many key residues are conserved, especially in the 2nd trans-membrane pore lining region, M2. The M2 region is responsible for ion selectivity, ion flux, and binding a wide range of non-competitive inhibitors (Alqazzaz et al., 2011). The aim of this work was to investigate the GLIC M2 region, especially His235 (or 11'), which has been proposed as essential in proton sensing linked to channel opening (Wang et al., 2012), and residues that are conserved across the Cys-loop receptor family. To probe the roles of specific amino acids in GLIC M2 trans-membrane domain, we substituted M2 residues lining the ion pore and M2 residues facing M3 trans-membrane domains. We generated over 40 mutations at various positions including those at Glu 222(-2'), Thr 226 (2'), Ser 230 (6'), Leu 232 (8'), Ile 233 (9'), Ala 234 (10'), Ile 236 (12'), Ala 237 (13') and Phe 238 (14') using site-directed mutagenesis, and tested their function using two-electrode voltage clamp as previously described (Alqazzaz et al., 2011). Our results showed that Ser6', Ile9' and His11' residues are very sensitive to subsitution with all substituents resulting in non-functional receptors. We conclude that His 11' has a role in channel opening/closing, and the residues Thr226, Ser230, and Ile233 also play an important role in receptor function.

Alqazzaz M, Thompson AJ, Price KL, Breitinger HG, Lummis SC (2011). Cys-loop receptor channel blockers also block GLIC. Biophysical journal 101(12): 2912-2918.

Wang HL, Cheng X, Sine SM (2012). Intramembrane proton binding site linked to activation of bacterial pentameric ion channel. The Journal of biological chemistry 287(9): 6482-6489.

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Functional Modulation of the ELIC by General Anesthetics and Alcohols

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ELIC is a prokaryotic pentameric ligand gated ion channel (pLGIC) homologous to Cys-loop receptors, which are molecular targets of general anesthetics and alcohols. Crystal structures of ELIC and its complex with the antagonist acetylcholine have been resolved recently, suggesting the possibility of using ELIC to understand the structural basis of anesthetic and alcohol modulation on pLGICs. However, the pharmacological profiles of ELIC for modulation by general anesthetics and alcohols have not been well defined. In this study, we characterized these profiles for ELIC expressed in Xenopus laevis oocytes with two-electrode voltage clamp techniques. We found that the ELIC current elicited by the agonist propylamine could be inhibited by both volatile and intravenous general anesthetics at clinically relevant concentrations. ELIC is also inhibited by ethanol and other n-alcohols with potency increasing with the number of carbons until n-nonanol, where inhibition is cut off. Alcohol modulation on ELIC is similar to that on GABAR-ρ1 but different from nAChRs, which are potentiated by ethanol. In addition, ELIC is inhibited by the benzodiazepine drug diazepam. In summary, ELIC shares some pharmacological characteristics of cation-conducting eukaryotic pLGICs and is a suitable model for the structural study of the actions of general anesthetics and alcohols on pLGICs. Supported by NIH (R01GM066358, R01GM056257, and R37GM049202).

3265-Pos Board B420

Alcohol and Anesthetic Binding to Pentameric Ligand-Gated Ion Channels Revealed in a Prokaryotic Model System

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